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Nicotinic Activation of CRH Neurons in Extrahypothalamic Regions of the Rat Brain

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Nicotine is known to have multiple effects on neuroendocrine, autonomic, and behavioral responses. Its neuroendocrine effect on the stress-responsive hormone, ACTH, depends on central pathways that act on corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus (PVN). Other CRH neurons throughout the brain also are involved in coordinating aspects of the stress response, but very little is known about the effect of nicotine on CRH neurons in extrahypothalamic regions that are involved in the autonomic and behavioral responses to stress. The current study sought to determine the extent of nicotinic activation of extrahypothalamic CRH neurons, since these neurons may be involved in mediating the central effects of nicotine. Freely moving rats were pretreated with a low dose of colchicine, infused with nicotine (0.045 mg/kg/30 s or 0.135 mg/kg/90 s, iv), and cardiac perfused 1 h later. Double-label immunocytochemistry identified the activated (positive for cFos protein) CRH neurons in limbic structures (bed nucleus of the stria terminalis [BNST] and central nucleus of the amygdala [CNA]), the dorsal raphe (DR), and Barrington's nucleus (BN); comparisons were made to the PVN. In all of these areas, nicotine activated CRH neurons in a dose-dependent manner, showing differential sensitivity and efficacy with respect to region. CNA CRH neurons were most responsive and were maximally stimulated by the low dose of nicotine (62% of CRH neurons were cFos+, compared to 10–27% of the CRH population in other regions, including the PVN). Although the BNST also was activated by the low dose, only the non-CRH+ neurons were involved; in contrast, 41% of the BNST CRH neurons responded to the higher dose. Nicotinic activation of DR neurons was dose-dependent, with 22% of the CRH neurons activated by the high dose. Few BN neurons were activated by the low dose of nicotine, but 26% of the CRH population responded to the higher dose. These results indicate that the effect(s) of nicotine on the brain may be mediated, in part, by the selective activation of specific extrahypothalamic regions containing CRH neurons that also are involved in autonomic and behavioral responses to stress. The large fraction of CRH neurons responding to the low dose of nicotine in the CNA suggests that this limbic region may be particularly important in mediating these CNS effects of nicotine.

Key Words: cFos; amygdala; bed nucleus stria terminalis; dorsal raphe; Barrington's nucleus; paraventricular nucleus.

Introduction

Nicotine is a psychostimulant with known effects on neuroendocrine, autonomic, and behavioral responses. Nicotine acts centrally within the brain to activate neuronal pathways that stimulate CRH neurons in the parvocellular region of the paraventricular nucleus (pcPVN) of the hypothalamus, with the sequential release of the stress-responsive hormone, ACTH, followed by glucocorticoids. This activation of the hypothalamo–pituitary–adrenal (HPA) axis by nicotine has been reported by a number of laboratories *(1–3)*. In addition to the role of parvocellular CRH neurons in the HPA-mediated stress response, other CRH neurons throughout the brain contribute to the coordination of the overall stress response *(4,5)*. Extrahypothalamic CRH neurons have been shown to be involved in responses to physical and, particularly, psychological stressors *(4,6)*. Many of the extrahypothalamic CRH regions also may be involved indirectly in neuroendocrine functions, since they project to the PVN. Thus, we sought to define the role of nicotine in the activation of extrahypothalamic CRH neurons, since these may contribute to the central nervous system (CNS) effects of nicotine.

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The following brain regions that contain CRH neurons were examined:

1. Limbic structures (the bed nucleus of the stria terminalis [BNST] and the central nucleus of the amygdala [CNA]),
2. The dorsal raphe (DR, B7 serotinergic region).
3. Barrington's nucleus (BN; M6 or pontine micturation region).

The limbic structures were chosen owing to their demonstrable involvement in HPA-independent stress responses *(4,7)*. The DR was selected because of its involvement in stress-related avoidance behavior *(8)*. In addition, the DR has significant input to both the CNA and the PVN *(9–11)*. DR–CNA connections are involved in HPA-independent stress responses, and DR–PVN pathways modulate HPA-dependent responses. BN was selected owing to its involvement in HPA-independent stress responses *(12)*. In addition, CRH neurons in BN appear to interact with neurons in the immediately adjacent locus ceruleus (LC; A6 noradrenergic region *[13]*); noradrenergic pathways from LC have a significant role in stress responses unrelated to HPA activation *(5)*.

Activation of these extrahypothalamic regions by nicotine may be involved in some of its prominent pharmacological actions. Nicotine administration has been shown to increase the startle response, decrease body temperature, and to have a dose-dependent effect on locomotor activity and heart rate in rodents *(14–17)*. Some of these behavioral and autonomic responses involve limbic structures and are similar to those seen in response to anxiogenic stimuli. For example, activation of the CNA has a role in acoustic startle and increased cardiac output *(7,9)*. Some appetitive behaviors associated with nicotine have been shown to be mediated by serotonergic neurotransmission originating in the DR *(18)*. Finally, LC activation is involved in nicotine-enhanced vigilance and arousal *(19)*. Each of these extrahypothalamic regions contains nicotinic cholinergic receptors, as indicated by the binding of nicotinic ligands *(20–22)*, and CRH neurons *(13,23,24)*. Therefore, these extrahypothalamic CRH regions may be involved in integrating the effects of nicotine on the brain, especially with respect to modulating stress-responsive pathways.

This investigation assessed the magnitude of the response of extrahypothalamic CRH-containing regions to nicotine. This was accomplished by measuring nicotine-induced expression of cFos protein in these regions. To determine both the number and fraction of CRH neurons activated by nicotine within each region, double-label immunocytochemistry for cFos and CRH was used. The percentage of activated CRH neurons (i.e., cFos+ and CRH+) within a region also was compared to the overall neuronal population responding to nicotine (total cFos+). Although it is accepted that cFos expression is a marker for neuronal activation by a stimulus or pharmacologic agent, the caveat must be made that cFos activation is not *a priori* evidence for mediation of a complex behavior by a specific neuronal population(s).

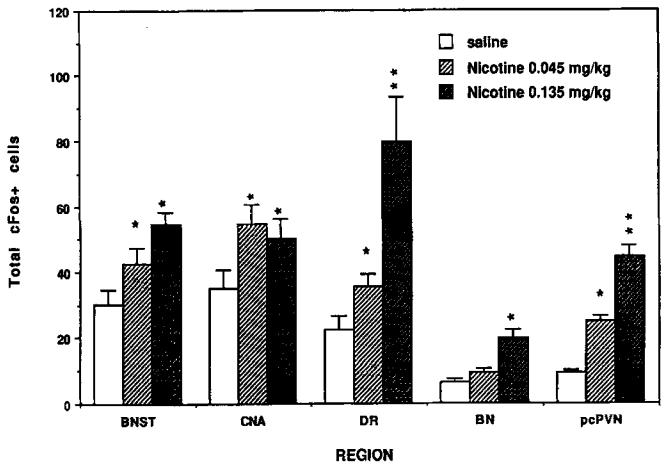

Fig. 1. Regional activation by nicotine. Alert, freely mobile rats, pretreated with a low dose of colchicine, were infused iv with saline (open bars) or nicotine (0.045 mg/kg [light bars] or 0.135 mg/kg [dark bars]) and cardiac perfused 60 min thereafter. Specific regions were counted bilaterally on 4–6 sections from each brain (3–5 rats/group); data from each treatment group were averaged and expressed as the mean (±SEM) number of cells in a unilateral region per section. Nicotine significantly increased cFos expression in all the CRH-containing regions examined. Most regions showed an increase in the number of cFos cells activated in response to the lower dose of nicotine. The exception was the LC, which became activated only by the higher dose of nicotine. BNST = bed nucleus of the stria terminalis; CNA = central nucleus of the amygdala; DR = dorsal raphe; BN = Barrington's nucleus; PVN = parvocellular region of the paraventricular nucleus. $*p < 0.05$ compared to saline; $**p < 0.05$ compared to lower dose. Comparisons of the number of cFos cells between regions were not performed because regions contain significantly different numbers of cells.

Results

The increase in the number of cFos+ cells by region is demonstrated in Fig. 1. As seen in the saline controls, cFos was constitutively expressed in the limbic structures (30 ± 4.5 and 35.2 ± 5.6 cells/section for BNST and CNA, respectively) and the DR (22.5 ± 4.2). Baseline levels of cFos in the BN (6.4 ± 1.12) and pcPVN (9.5 ± 0.6) were very low. Nicotine significantly increased cFos expression in all of these CRH-containing regions, with all areas (except BN) showing an increase in the number of cFos+ cells responding to the low dose of nicotine (0.045 mg/kg; $p < 0.05$). Compared to saline, these increases ranged from approx 1.5-fold above baseline in the limbic structures (42.5 ± 5.1 total cFos+ cells/section in BNST and 54.4 ± 6.0 in CNA) to 2.5-fold in the pcPVN (24.9 ± 1.5 cells/section). BN was activated only by the higher dose of nicotine (0.135 mg/kg), which produced a twofold increase in cFos expression (19.9 ± 2.6 cells/section). The higher dose of nicotine did not stimulate an additional increase in the number of cFos+ cells in either limbic region (BNST [54.4 ± 4.1 cells/section] or the CNA [50.2 ± 5.7]). In contrast, cFos expression increased further in the DR (79.6 ± 13.9) and the pcPVN

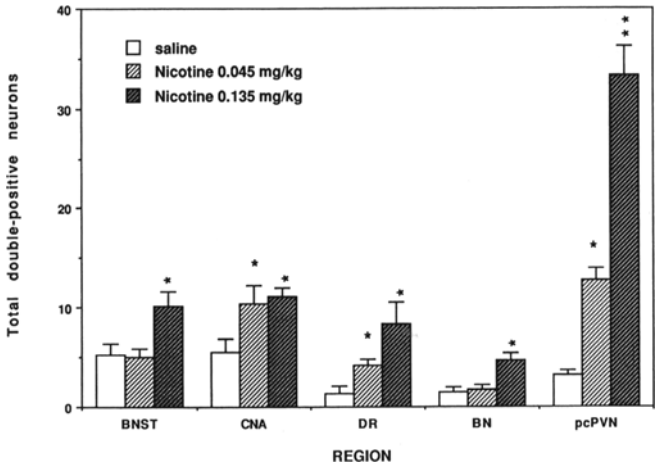

Fig. 2. Specific activation of CRH-containing neurons by nicotine iv. In alert, freely mobile rats, pretreated with a low dose of colchicine (7 μg/10 μL/100 s, 16–18 h previously), nicotine significantly increased cFos expression in CRH neurons. $*p < 0.05$ compared to saline; $**p < 0.05$ compared to lower dose. Comparisons between regions in the number of cFos+/CRH+ neurons were not performed because regions contain significantly different numbers of CRH+ neurons.

(44.6 ± 3.2). Thus, regional differences in responsiveness to nicotine were observed.

In addition to differences in the regional induction of cFos expression by nicotine, the response of CRH+ neurons to nicotine (neurons positive for both cFos and CRH) showed regional specificity. As shown in Fig. 2, CRH neurons in the limbic structures were constitutively activated (5.2 ± 1.1 and 5.5 ± 1.3 cells/section in BNST and CNA, respectively), and the low dose of nicotine did not stimulate further activation in the BNST (5.0 ± 0.9). In contrast, the CNA CRH neurons were maximally stimulated by the low dose of nicotine (10.4 ± 1.8); this twofold induction over basal levels was not increased by the higher dose (11.1 ± 0.9). Photomicrographs in Fig. 3 illustrate this activation of CRH neurons in the CNA. The punctate background in the photomicrographs is the result of the presence of CRH+ fibers and terminals from the extensive CRH+ afferents to

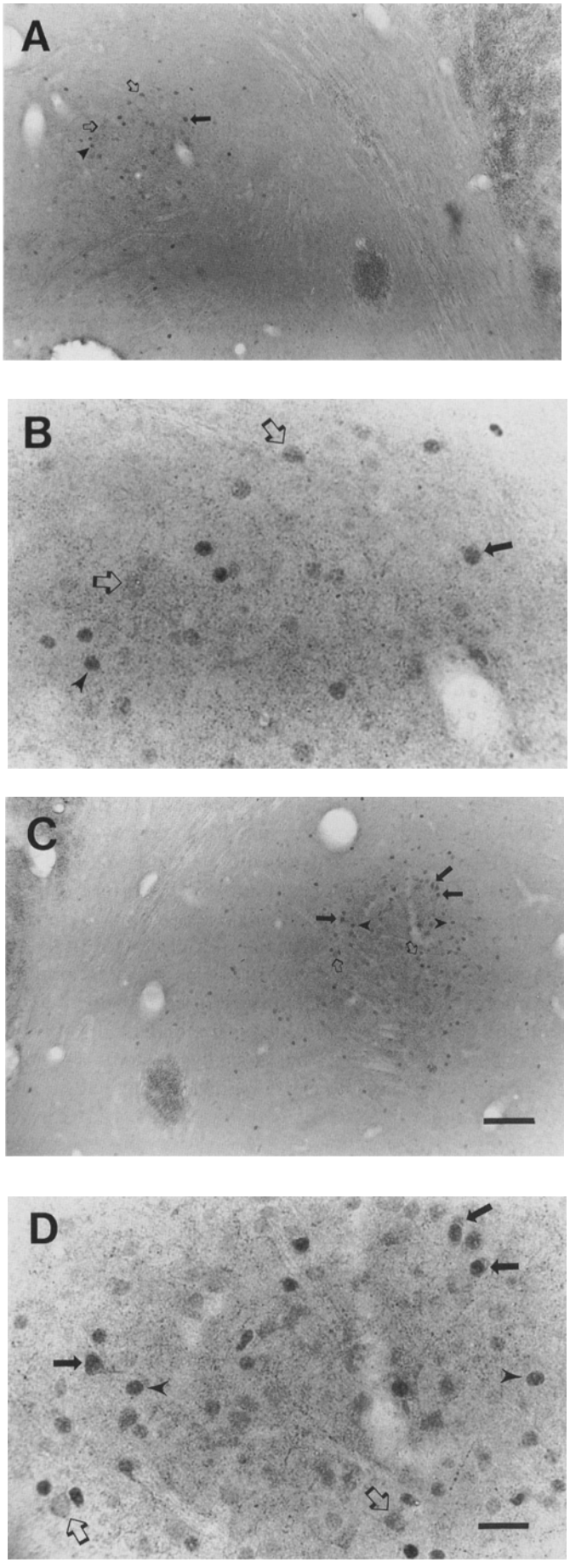

Fig. 3. *(right)* Photomicrographs of the specific activation of CRH neurons in the CNA by nicotine. Rats were cardiac perfused 1 h after nicotine was administered and the brain tissue stained by double-label immunocytochemistry for cFos (nuclei; arrowhead) and CRH (cytoplasm; open arrow); double-labeled neurons (cFos+ and CRH+) are identified by a closed arrow. Panels **A** and **B** = saline controls; panels **C** and **D** = nicotine 0.045 mg/kg over 30 s iv. Low-power magnification bar = 100 μm (panels A and C); high magnification bar = 25 μm (panels B and D); the same neurons are identified in both the low- and higher-magnification photomicrographs for each treatment group. The punctate appearance of the background is attributable to the fibers and terminals of the extensive CRH afferents to the CNA. When visualized in color and through focus on a microscope, CRH+ cell bodies are sufficiently distinct for quantitation.

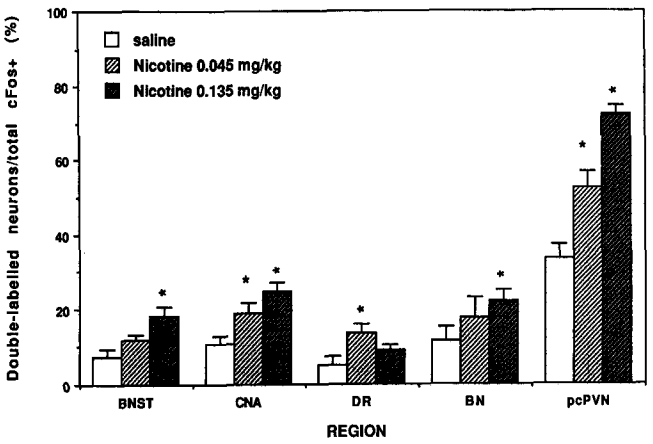

Fig. 4. The contribution of CRH neurons to the total population of cells activated by nicotine. The proportion of double-labeled neurons in the total cFos population increased in the CNA, DR, and PVN in response to the low dose of nicotine (0.045 mg/kg, light bars). The BNST and BN CRH populations were activated by the higher dose of nicotine (0.135 mg/kg; dark bars), whereas the CNA CRH neuronal population was already maximally stimulated. The apparent decrease in DR CRH activation at the higher dose was owing to a large increase in the number of unidentified cells in this region, rather than a true decrease or inhibition of cFos activation in the CRH neurons.

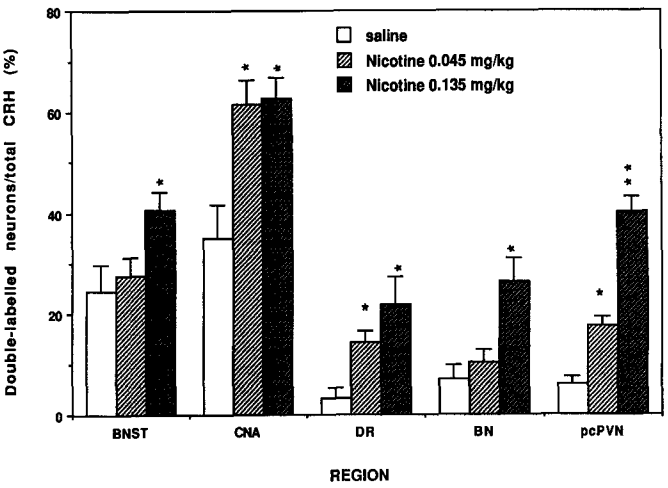

Fig. 5. The fraction of the CRH+ population that was activated by nicotine. In response to the low dose of nicotine (light bars), a significant activation of CRH neurons was seen in the CNA, DR, and pcPVN, compared to saline controls (open bars). At the higher dose of nicotine (dark bars), CRH activation in each of the other regions increased significantly, with the exception of the CNA CRH neurons, which were maximally stimulated already.

the CNA. The low dose of colchicine used in these experiments is sufficient to visualize (and count) CRH+ cell bodies when observed through focus and in color with a microscope. In black and white photomicrographs, the contrast between the CRH+ neuronal cytoplasm and the CRH+ terminals or fibers is less well-defined.

Similar to the maximal activation of CRH+ neurons in the CNA by nicotine 0.045 mg/kg, in the DR, the significant activation of CRH neurons by this lower dose (4.2 ± 1.6 compared to 1.4 ± 0.7 for saline) was not further increased by the higher dose (8.3 ± 2.2). CRH neurons in BN, unresponsive to the low dose of nicotine, showed a threefold increase in response to the higher dose (4.6 ± 0.8 compared to 1.5 ± 0.5 for saline). Finally, the CRH neurons in the pcPVN showed significant, dose-dependent responses, with a fourfold increase in response to low-dose nicotine (12.7 ± 1.2) and 10-fold to the higher dose (33.3 ± 2.9), compared to baseline levels (3.2 ± 0.5). These findings indicate that CRH-containing neurons show regional differences in responsiveness to nicotine; only CNA, DR, and pcPVN responded to the lower dose. Moreover, only CNA CRH neurons were maximally responsive to the lower dose of nicotine.

Figure 4 shows the fraction (expressed as a percentage) of the activated cells in each region (total cFos+) that was attributable to CRH neurons (positive for both cFos and CRH). In the BNST, CRH neurons comprised 7 ± 2.3% of the constitutively activated neurons, a ratio that did not change in response to the low dose of nicotine (11.8 ± 1.4%), but did increase significantly at the higher dose (18.4 ± 2.2%). Similar responses were seen in BN, where only the higher dose of nicotine resulted in a significant increase in the fraction of activated neurons that contained CRH (21.8 ± 3.3% compared to 11.5 ± 3.7% in saline controls). In contrast, in the CNA the maximal increase (twofold) was seen in response to the lower dose. In the pcPVN, over 50% of the activated neurons were CRH+ in response to 0.045 mg/kg nicotine, and this number rose to over 70% at the higher dose. Since CRH neurons comprise approx 80% of the neurons in this region *(24)*, a response in >70% of the pcPVN neurons may represent near-maximal stimulation. The data indicate that the CRH neurons in the CNA, DR, and PVN comprised 15–50% of the neurons responding to lower dose nicotine.

In the DR (Fig. 4), the apparent decrease in the number of CRH neurons activated in response to the higher dose of nicotine was not a true reduction (i.e., inhibition of Fos expression). Rather, it was owing to a much greater increase in total number of activated cells (total cFos+; Fig. 1) compared to the slight increase in activation of CRH+ neurons (Fig. 2). More CRH neurons might have become activated by doses of nicotine greater than 0.135 mg/kg, but behavioral responses (suggestive of nonspecific stimulation) become apparent at higher concentrations *(25)*.

Figure 5 shows the fraction of the CRH neuronal population in each region that was activated by nicotine [(cFos+/CRH+ neurons)/CRH+ neurons; expressed as a percentage]. This fraction provides an index for comparing the relative nicotine responsiveness of the CRH neurons in these regions. At the low dose of nicotine, the response in the CNA was most pronounced with >60% of the CRH+ neurons activated. At the higher dose, approx 22–26% of the CRH neurons in the DR and BN were activated, whereas

over 40% of the CRH neurons in the BNST and pcPVN were positive for cFos. Dose dependency was most pronounced in the pcPVN, where the percentage of activated CRH neurons significantly increased from 17% (low dose) to 40% ($p < 0.01$ compared to low dose nicotine).

Discussion

CRH has a well-established role in integrating the endocrine, autonomic, and behavioral responses to stressors *(4)*. The CRH neurons are located in regions of the brain that also contain other neuropeptides and neurotransmitters (e.g., norepinephrine and serotonin) involved in these responses. Connections between the regions investigated in this report involve both CRH neurons and these other neurons. For example, there are significant CRH inputs to BN from both the PVN and limbic structures *(9,24,26)*. Conversely, there are CRH projections from BN *(27)*, as well as serotinergic inputs from the DR and CNA *(9)* and noradrenergic inputs from the LC *(28)*, to each of the other CRH-containing nuclei. The reciprocal circuits between these regions comprise some of the major pathways involved in the stress response, suggesting the importance of both the intrinsic CRH populations and the extrinsic CRH inputs to these areas. Activation of these CRH neurons by nicotine may be involved in some of the prominent pharmacological actions of the drug. In the current studies, we have shown that there are significant regional differences in the responsiveness of CRH neurons within these extrahypothalamic regions to nicotine.

Central Nucleus of the Amygdala (CNA)

Within the amygdala, the central nucleus is a major site of CRH neurons, and evidence suggests that these specific neurons may play an important role in mediating responses to stress *(9)*. Amygdaloid neurons have been shown to be activated by a variety of stressors, including anxiogenic stimuli *(4,9,29)*. In addition, after immobilization stress, there is a significant increase in the expression of cFos in the CNA, primarily within CRH+ neurons. Intracerebronuclear (icn) injections of CRH into the CNA have been shown to cause increases in cardiac output and systemic blood pressure *(30)*, and to increase locomotion within familiar environments, but decrease exploration within unfamiliar places *(9)*. Intra-CNA CRH injections also have been shown to enhance stress-induced freezing behavior *(31)*, as well as the proconflict effect observed in the Geller-Seifter conflict procedure *(32)*. Several of these effects (e.g., increased cardiac response *[30]*) have also been shown to be blocked by icn injections of the CRH antagonist, α-helical CRH, as is the "defeat" response exhibited by rats placed into territories occupied by more dominant rats *(7,33)*. In addition, bilateral lesions of the CNA result in decreased ACTH secretion after immobilization stress and block both stress-induced freezing and the cardiovascular responses induced when threatening stimuli are presented *(9,34)*. These studies suggest that CRH release within the CNA, possibly from CRH neurons intrinsic to the CNA, may be involved in mediating the autonomic and behavioral responses elicited by anxiogenic stimuli and stressors. Moreover, because some CRH neurons in the CNA project directly to the pcPVN *(35)*, CRH release in the CNA might affect HPA responsiveness to stressors as well.

In regard to the known effects of nicotine on the CNA, cFos expression in CNA has been reported in response to injections of nicotine *(36,37)*. However, the chemical phenotype(s) of the neurons that respond to nicotine has not been identified. It has been shown that acetylcholine induced CRH release from the amygdala in vitro, and this effect was blocked by the nicotinic cholinergic antagonist, mecamylamine *(38)*. Taken together with the current finding of a 30% increase in the number of CNA CRH neurons stimulated by the low dose of nicotine (above the response to saline; Fig. 5), these studies suggest that nicotine might stimulate the release of CRH from CNA neurons. Thus, CRH may function as an intrinsic neurotransmitter by mediating some of the effects of nicotine on other amygdaloid neurons, including those in the CNA. In addition, the CNA CRH neurons may modulate neurons in the other regions studied herein, since they project to each of them. Compared to the other regions, CNA CRH neurons are more responsive to nicotine (30% incremental activation by low-dose nicotine, compared to 3–11% for the other regions; Fig. 5). Therefore, nicotinic stimulation of CNA CRH neurons may be a central mediator of neuronal responses to nicotine.

Bed Nucleus of the Stria Terminalis (BNST)

Anatomically, the BNST can be considered an anterior extension of the limbic system and the amygdaloid complex in particular *(39,40)*. At the cellular level, both the BNST and amygdala contain similar peptidergic neurons (CRH, neurotensin, enkephalin, somatostatin, substance P, galanin) and receive comparable afferent input. They also send reciprocal inputs to each other. In addition, there are reciprocal connections between these limbic structures and monoaminergic regions of the brainstem (including the LC). Direct catecholaminergic–CRH synapses have been demonstrated in the BNST and CNA *(41–43)*. Finally, both limbic structures project to the pcPVN *(35,39)*.

Despite these similarities between the BNST and the CNA, the data reported herein demonstrated a difference in the responsiveness of the CRH neurons in these regions to nicotine. In the CNA, both overall neuronal activation (total cFos; Fig. 1) and fractional CRH activation (Fig. 5) were maximally stimulated by the lower dose of nicotine. With respect to overall neuronal activation, the BNST showed a statistically similar pattern. However, the cells responding to the low dose of nicotine were not CRH neurons, since there was no increase in the number of double-

labeled neurons over baseline (Fig. 2). Recruitment of BNST CRH neurons occurred only at the higher dose of nicotine, with a 16% increase above the response in saline controls (Fig. 5). Therefore, it appears that there is a distinct difference in the response of these limbic CRH subpopulations to nicotine. A similar divergence between the BNST and the CNA has been seen in response to chronic corticosterone *(44)*. In that report, CRH levels in the BNST were unaffected, whereas those in the CNA rose slightly, implicating a difference in synthesis or release between the related limbic regions.

Barrington's Nucleus (BN) and Locus Ceruleus (LC)

To our knowledge, an effect of nicotine on BN has not been demonstrated previously. This stress-responsive nucleus projects to the PVN, as well as to the other extrahypothalamic CRH regions of the brain *(26,30)*. Acute immobilization stress (60 min) or footshock stress enhance CRH mRNA in this nucleus *(12,45)*. In addition, BN neurons sustain their cFos responsiveness to repeated (10-d) restraint stress, whereas cFos activation in the PVN desensitizes, indicating that activation of this nucleus by stress is independent of the HPA *(46)*.

CRH neurons in the BN may have a putative influence on the noradrenergic neurons of the LC, since tyrosine hydroxylase-positive dendrites from the LC extend into BN *(13,26)*. This influence may be important, because the LC is the major source of norepinephrine in the brain and is extensively involved in the CNS stress response (reviewed in ref. *28*). In particular, LC activation after iv nicotine administration is of interest because of the role of noradrenergic neurons in HPA-independent responses to stress *(5)*. There are a number of studies investigating the role of CRH in the LC response to stressors. The concentration of CRH in the LC is increased by chronic unpredictable stress and decreased by administration of the anxiolytic, alprazolam *(47,48)*. Activation of the LC by hemodynamic stress mimics that seen in response to local injections of CRH and is antagonized by α-helical CRH *(28,49,50)*. Since there are no CRH neurons intrinsic to the LC *(13,28)*, one source of CRH in the LC is BN neurons that are in close proximity to the dendrites of the noradrenergic neurons. Although direct synaptic contact between these neuronal types has not yet been demonstrated *(13)*, CRH does stimulate the release of norepinephrine *(50,51)*. Additional sources of CRH in the LC are the CRH inputs from limbic structures and/or the pcPVN; these also may mediate, in part, the effects of nicotine on LC noradrenergic neurons. Previous studies have shown that the LC itself is relatively unresponsive to nicotine delivered either iv or intraparenchymally, as measured by cFos activation or electrophysiology *(25,52,53)*. Therefore, CRH neurons, both adjacent to and distant from the LC, may play an active role in nonHPA-mediated responses to nicotine by modulating the activity of this major noradrenergic nucleus.

Dorsal Raphe (DR)

Although the DR is the major source of serotonin in the brain, only 40–48% of its neurons are serotonergic *(54)*. CRH neurons also are intrinsic to the DR, and although their precise function has not been elucidated, the DR has been shown to project to the CNA and the PVN *(11,55)*. In fact, some of the CRH axons from the DR specifically synapse with other CRH neurons within the CNA *(9)*. In addition, direct serotonergic input from the DR to PVN CRH neurons appears to be involved in serotonergic modulation of the HPA axis *(10,56)*. Although there is no evidence to our knowledge that nicotine acts directly on DR neurons to stimulate the release of CRH or serotonin, there are reports that nicotine stimulates the release of serotonin in the cortex in vivo *(18,57)* and in the striatum in vitro *(58)*, possibly by acting on DR neurons. It was shown in the present investigation that the CRH population in the DR, like those in the CNA and pcPVN, was responsive to a low dose of nicotine. Therefore, the extrahypothalamic CRH neurons in the DR also may be actively involved in the response(s) to nicotine, both directly and indirectly, through modulation of both the CNA and PVN.

Nicotinic Cholinergic Receptors (NAchRs)

The presence of NAchRs in each of the nuclei under investigation supports the concept that each nucleus can be directly responsive to nicotine. ^{3}H-nicotine binding in the BNST, CNA, LC, and DR has been documented *(20–22)*, as well as binding in the neuropil surrounding the PVN *(59)*. Furthermore, ^{125}I-αbungarotoxin binding occurs in the each of these regions and within the PVN *(20,21)*. In addition, chronic nicotine administration has been shown to result in a dose-dependent increase in NAchR number (i.e., ^{3}H-nicotine binding increased) in the CNA, DR, and LC *(22)*. However, there are no reports to our knowledge that specifically demonstrate NAchRs on CRH or on any phenotypically identified neurons. Without such evidence, the mechanism of action of nicotine on CRH neurons remains undefined.

These results indicate that the effect(s) of nicotine on the brain may be mediated, in part, by the selective activation of specific extrahypothalamic regions containing CRH neurons that also are involved in autonomic and behavioral responses to stress. The large fraction of CRH neurons responding to the low dose of nicotine in the CNA suggests that this limbic region may be particularly important in mediating these CNS effects of nicotine.

Materials and Methods

Animals

All procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and with the approval of the Animal Care and Use Committee of the Minneapolis Medical Research Founda-

tion. Adult male Holtzman rats (250–300 g; HSD, Madison, WI) were individually housed, maintained on a 12-h light/dark cycle (lights on at 6 AM), fed standard rat chow and water ad libitum, and prehandled for 4–5 d prior to surgery. Rats were equipped with a chronic guide cannula in the lateral ventricle under ketamine:xylazine anesthesia and allowed to recover for 5–7 d. The jugular vein was cannulated under InnovarVet anesthesia (10 mg/kg body weight, ip; Pitman Moore, Mundelein, IL) 48 h prior to the experiment *(60)*. Intravenous (iv) injections were accomplished without handling or restraining the rats, since the jugular cannula was connected via a tether to the outside of the cage.

The purpose of these experiments was to compare activation of CRH neurons in the PVN by iv nicotine with activation of extrahypothalamic CRH neurons in the same rat. Since pretreatment with colchicine is essential for visualization of CRH in neuronal cell bodies of the PVN, a low dose of colchicine (7 μg in 10 μL, given over 100 s; *61*) was injected into the lateral ventricle 16–18 h prior to the beginning of the experiment. Although much higher doses of colchicine are routinely used for immunohistochemical studies (100–200 μg, 48–72 h prior to cardiac perfusion *[13,62]*), such doses activate cFos expression and elevate plasma corticosterone *(6,63)*. In contrast, the low dose of colchicine used in this study allows visualization of CRH neuronal cell bodies with minimal induction of cFos expression in the PVNs of control animals *(25,64)*, and minimal elevation of plasma ACTH *(61)*. In addition, this dose of colchicine has little effect on the synthesis and processing of neurohormonal precursors *(65)*. Therefore, a modification of the low-dose method was used in which rats were equipped with chronic guide cannulae in the ventricle, and intracerebroventricular (icv) injections were carried out under minimal stress conditions, as previously described *(25,66)*.

On the morning of the experiment, alert, freely mobile rats were injected iv with saline (Sal; $n = 3$) or nicotine (Nic, 0.045 or 0.135 mg/kg body weight; $n = 5$) 1 h prior to the onset of cardiac perfusion. A constant 0.09 mg/kg/60 s rate of infusion was used; a 30-s infusion delivered 0.045 mg/kg and a 90-s infusion delivered 0.135 mg/kg. We have reported previously that these infusions dose-dependently increased cFos expression in specific regions of the brain and stimulated the secretion of ACTH *(25,67)*.

Animals were lethally anesthetized with Nembutal (100 mg/kg body weight, ip) and then perfused transcardially on ice, as previously described *(25)*: initially with 2% sodium nitrate in saline (to vasodilate and flush out the blood), followed by 300 mL ice-cold fixative containing 2.5% acrolein and 4% paraformaldehyde in 0.05 *M* phosphate buffer, pH 6.8. Effluent was immediately neutralized with 10% sodium bisulfite in water. After fixation, the brain was rapidly removed and infiltrated overnight with 20% sucrose in phosphate buffer at 4°C, and then rapidly frozen and serially sectioned at –18°C.

Immunocytochemistry

Free-floating, 25-μm, serial sections were processed for double-label immunocytochemistry with cFos and CRH antibodies, as previously described *(25)*. Briefly, the initial incubation was with antibody to cFos protein (1:3000, 48 h at 4°C; specific for the N-terminal peptide of the cFos protein and noncrossreactive with other members of Fos family; Oncogene Science Inc., Uniondale, NY), followed by visualization with nickel sulfate-intensified diaminobenzadine (DAB), resulting in a blue-black color in the nucleus. The second incubation was with CRH antibody (1:60,000, 48 h at 4°C; noncrossreactive with POMC-derived peptides or other adrenocorticotropin secretogogs *[68]*), followed by visualization with DAB, resulting in a yellow-brown color in the cytoplasm. Intensification of the CRH immunostaining in cell bodies was minimized to allow identification of cFos+ nuclei within double-labeled neurons. This occasionally resulted in sections in which the level of staining of CRH in the fibers and terminals of the background approximated that within the soma (e.g., the CNA). However, with the ability to focus through the tissue with a microscope and the visual aid of gradations in color, accurate quantitation was possible.

The landmarks for identification of nuclear boundaries were based on those delineated in the atlas of Paxinos and Watson *(69)*. The pcPVN was, by definition, the CRH-positive region in the dorsomedial PVN, since it contains over 80% of the CRH neurons in the PVN *(24)*. The quantitated region of the BNST was medial to the ventral tip of lateral ventricle and dorsal to the anterior commissure. The CNA was dorsal to the optic tract, lateral to the stria terminalis, and medial to the ventral tip of the internal capsule. The DR was in the midline, ventral region of the pontine central gray. The BN was ventromedial to the LC and medial to the mesencephalic nucleus of the trigeminal nerve. The CRH neurons in the parabrachial nucleus and the dorsal vagal complex were not included in the analyses owing to the faint intensity of CRH staining in these regions. Similar variation in CRH staining intensity between brain regions has been described previously, even with much higher doses of colchicine *(24)*.

Data Analysis and Statistics

Brain regions of interest were analyzed bilaterally in 4–6 sections (representative of the anterior, middle, and posterior regions of a specific nucleus) from each brain ($n = 3$–5/treatment group), with counting performed blindly on coded slides. Counts were made of:

1. Activated cells (cFos+ nucleus).
2. CRH neurons (CRH+ cytoplasm).
3. Double-labeled neurons (cFos+/CRH+) in each section.

The number of activated CRH neurons (cFos+/CRH+) compared to the fraction of activated cells in each section that were cFos+ was calculated [(cFos+/CRH+)/cFos+]. The percentage of activated CRH neurons compared to the total number of CRH+ neurons/section also was calculated ([cFos+/CRH+]/CRH+). Data from each treatment group were averaged, and results were reported as the mean ± SEM of the number of cells in a unilateral region per section. Statistical analysis was performed with SYSTAT and results were considered significant at $p < 0.05$.

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